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May 20 1943

I hereby recommend that the thesis prepared under my supervision by William H. Hesselbrock

entitled Newer Aspects Of The Biology And Morphology Of Bacterium Tularense.

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

Form 668—G. S. and Ed.—1M—7-37

NEWER ASPECTS OF THE BIOLOGY AND MORPHOLOGY OF BACTERIUM TULARENSE

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1943

by

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A.B. University of Cincinnati 1936

M.Sc. University of Cincinnati 1939

Newer Aspects of the Biology of Bacterium Tularense

Part I Antigenicity

Historical

The association of inagglutinability with nonmotility appears to have been noticed first in B. typhosus by Malvoz (1). He believed that his experimental treatment of the bacilli removed their flagellated envelope and that both phenomena were the result of the loss of these structures.

Nicolle and Trenel (2) studied variations in agglutinability of the agglutinogenic power of B. typhosus and certain other organisms. From one of these which they described as "similar to B. typhosus", they isolated motile and non-motile strains, prepared serums with both, and carried out cross-agglutination tests. The "motile" serum agglutinated the motile, but not the non-motile form; whereas the "non-motile" serum agglutinated neither one. These results with the non-motile form of the organism stand in contrast with later observations. The probable explanation lies in the lack of delicacy of the technique in use at that time. No description of technique is available but workers of that period used one to two hours incubation at room temperature for agglutination tests. Thus the minute and very slowly forming clumps of the non-motile forms would not have occurred in this short time.

In 1903, Smith and Reagh (3) described the presence of two different agglutinins occurring in the blood serums of

rabbits immunized against B. cholera suis A. When a culture of the hog-cholera bacillus belonging to the motile race was exposed to the action of serum from an animal previously inoculated with motile bacilli it was noticed that large, loose, flocculi appeared soon after beginning the experiment, whereas, if serum from a rabbit immunized against the non-motile bacillus was mixed with this motile culture the clumps appeared fine and powdery to the naked eye and formed quite slowly. It was also observed that a longer and more intense immunization period was required to obtain an active serum against the non-motile form. It was subsequently demonstrated that the loose flocculi were produced by a flagellar agglutinin, whereas the fine clumps were attributed to a body agglutinin. The separation of these substances was effected by means of absorption experiments. The identity of the "body" agglutinins of "motile" and "non-motile" serums was thus established as was also the non-identity of the flagellar and body agglutinins coexisting in "motile serum.

About the same time Joos (4) described two agglutinins, designated by him A and B, in the serum of animals immunized against B. typhosus. He also described two complementary agglutinable substances of the bacilli themselves, which he called A and B agglutinogens. Joos was able to demonstrate these different bodies concerned in typhoid agglutination by virtue of their unlike reactions toward heat and the different appearance of the clumps to which they gave rise. According to him a temperature of 60° to 62°C. maintained for an hour was sufficient

to destroy the A agglutinable substance and the B agglutinin, while the A agglutinin and the B substance remained unaffected. At ordinary temperatures the A agglutinin showed a specific affinity for the A substance, their union being characterized by the formation of coarse, rapidly forming flocculi. The B agglutinins likewise combined, but the clumps to which they gave rise were fine and formed comparatively slowly.

In 1904, Beyer and Reagh (5) used the term "somatic", in place of the term, "body", in referring to non-motile agglutination. Working with the hog-cholera bacillus they were able to effect a further differentiation of the flagellar and somatic agglutinins by means of heat. They found that a temperature of 70°C. for at least twenty minutes impaired the somatic agglutinin of the serum and the flagellar agglutinable substance of the hog-cholera bacillus. It left nearly intact the flagellar agglutinin of the serum and the somatic agglutinable substance of the bacilli. They found that a higher temperature was required to differentiate the flagellar and somatic bodies of the hog-cholera bacillus than was necessary in separating the corresponding substances from the typhoid bacillus and that a temperature of 70°C., which was sufficient to destroy the agglutinating power of motile hog-cholera bacilli, did not affect their power of generating flagellar agglutinins in the animal body.

During the investigation into the nature of serodiagnosis in typhus fever it was observed by Weil and Felix that although Proteus Vulgaris X_{19} and Proteus Vulgaris X_2 each

reacted specifically with the serums of active typhus patients or convalescents, such reaction was by no means equally marked, the X_{19} strain being agglutinated by very much higher dilutions of serum than the X_2 strain. On the other hand, immune serums artificially produced in rabbits were found to agglutinate both X_{19} and X_2 strains in equal titers. The nature of the agglutination was dissimilar, however. That brought about by the serums of patients showed itself as small, firm, granular, slow-forming flakes. With homologous immune serum agglutination occured rapidly and was characterized by the appearance of large, voluminous, loose, coarse flocculi. Furthermore, although serums from typhus patients agglutinated only X strains, the homologous immune serum often agglutinated to titer not only the X strains but also ordinary saprophytic strains of Proteus.

So striking was the difference between the effects of the two types of serum on various species of Proteus that it generally concluded that the agglutinins of typhus did not owe their origin to the antigens of the X strain. By proving that there existed two different types of receptors in the antigenic apparatus of X strains Weil and Felix did much to clear up the question. From the character of their colonies the X strains were found capable of differentiation into H (Hauch-hazy) and O (Ohne Hauch-clear) forms, of which the H form possessed two receptors, the O form only one. The O form evoked the small-flaking agglutinins which reacted only with the X strains, whereas the H receptor formed the large-flaking agglutinins that

reacted with the X strains as well as with ordinary saprophytic strains of Proteus. It was shown that in serums from typhus patients only O agglutinins (small-flaking) occurred, whereas in immune serums produced in rabbits against the X strains H agglutinins were present in addition.

Serologically considered there was great difference between the H and O forms. Immune serum of the O form (OX_{19} or OX_2) contained only one agglutinin which, reacting specifically with the bacteria, agglutinated them in small flakes (O agglutination). Immune serum of the H form, on the other hand, possessed two agglutinins, a specific small-flaking O agglutinin and a non-specific, large-flaking H agglutinin that reacted with both homologous and heterologous bacteria. Therefore, the antigenic structure of the O form consisted of one type of receptor; that of the H form of two types. The serum of typhus patients is in all respects identical with an immune serum produced by OX_{19} (containing only O agglutinins). Since homologous immune serum produced by the H form contained both O and H agglutinins this fact accounted for the difference between the behavior of serums from typhus patients and that of artificial immune serum to Proteus X organisms. It was later demonstrated that all strains of Proteus were equipped with double "binding groups" and that in the entire Proteus genus specific agglutination was effected by the O receptors, group agglutination by the H receptors.

Sachs (8), in 1918, discovered that H X strains when heated to 80°C. behaved exactly like O forms of these strains and decided that the H receptors were thermolabile and the O receptors thermostable. At about the same time Felix and Mitzenmacher (9) showed that if the H forms of Proteus were heated for two hours at 100°C. the H receptors were so affected that the heated antigen acted like an O form obtained by culture.

Weil, Felix, and Mitzenmacher (10) showed that all bacteria of the typhoid-paratyphoid-enteritidis group possessed the double type of receptor. Subsequently the terms H and O were superseded by others, these depending upon the inherent qualities of the receptors themselves. Thus the use of labile instead of H and stable instead of O became general. At the same time the agglutinins corresponding to the two types of receptors were named large-flaking (interacting with labile receptors) and small-flaking (interacting with stable receptors), hence the terms labilotrophic and stabilotrophic.

The phenomena noted above were demonstrated to be true for many bacteria, including B. typhosus and B. enteritidis as observed by Goyle (11,12), the Salmonella group as studied by Andrewes (13), and later elaborated by White (14), and for Vibrio cholerae as observed by Baltenau (15).

Due to the resemblance of the H and O forms of Weil and Felix to the S and R forms of many other intes-

tinal bacteria as developed by Arkwright, it might be well to ascertain to what extent the anology holds true. Arkwright and Goyle (16) observed in B. typhosus, B. enteritidis, and B. dysenteriae, antigens regarded as analogous to H and O. They showed that these two antigens could be demonstrated by heating an emulsion at 100°C. for ten minutes after which the heated emulsion absorbed the S agglutinins but not the H and thus could be used for obtaining a pure agglutinating serum. They also obtained variants containing only one antigen which might be either the R (H) or the S (O) according to their view.

These conclusions were in opposition to the trend of other evidence. White (17) showed that the smooth culture normally contained both H and O antigens, of which the former was more obvious in the tests with Salmonella. In some freshly isolated Salmonella cultures, and in those grown on phenol agar, the O property was predominant. Roughening was therefore concerned with a fundamental change in the O antigen. O smooth became O rough, which was quite a different antigen. Often rough cultures showed a sufficient amount of "smooth O antigen" to absorb all or part of the "smooth O agglutinin" from a smooth serum. However, a truly smooth culture had no rough O antigen. White also showed that roughening was associated with a reduction or total loss of the H antigen and that that change was permanent. Thus White brought the serologic changes involved in

the transformation of the H into the O type into a closer agreement with those of the S into the R type.

Goyle (18) studied strains of B. enteritidis Gaertner and B. typhosus by means of direct agglutination and agglutinin absorption and characterized the types as follows:
Smooth Form Normal: containing two antigens (similar to those of the H culture of Weil and Felix), the heat labile H (N or S) and the heat stable O, but no rough antigen (r).
Smooth Form Variant: containing ordinarily only a single antigen O (heat stable) occasionally also some H (N or S) and less frequently some R antigen.
Rough Variant: containing mainly the heat stable R antigen but often some of the heat labile, smooth, H antigen found in the normal smooth cultures and sometimes some O antigen.

To summarize, these results show that the H antigen of Weil and Felix is identical with the normal (N) or S form and dissimilar to the R form. Thus there is one heat labile antigen (S, H, or N, all synonymous) but there are two heat stable antigens, O and R. The inference is obvious. The difference in the proportions in which these antigens are present in any given infectious agent determine the character and scope of any serological diagnosis.

The earliest attempt to prepare a pure O antigen by chemical means was apparently made by Bien and Sonntag (19), who used alcoholic suspensions of Proteus for use in the diagnosis of typhus fever. Braun (20) found that cultivation on agar containing one tenth per cent phenol suppressed the smooth form (H antigen) but permitted the O forms to

grow. The organisms failed to develop flagella. Weil and Felix (21) noted that the H antigen was destroyed by the action of alcohol. This was confirmed by Bien (22), who used alcoholic treated cultures of Proteus OX_{19} and X_{19} for use in the Weil-Felix reaction.

Orcutt (23) worked with hog cholera (Maryland strain) and observed that certain of the bacilli lost their flagella and became non motile. With this change there was a concomitant change in their serologic behavior. Orcutt concluded from this that the flagella possessed an individual antigenic property. He separated the flagella from the bodies of the bacilli by vigorous shaking with glass beads and subsequent separation of the two by centrifuging. By means of this flagellar suspension he prepared a strictly flagellar antiserum and concluded from this that a specific separate antigen resided in the flagella. He also proved that the reaction of the flagella with its specific antiserum is an agglutination reaction and not a precipitin reaction, thus ruling out the criticism that the flagellar fraction might actually be, or contain, soluble substances from the bacteria themselves.

Arkwright (24), who worked with members of the colon-typhoid-salmonella group, confirmed the macroscopic evidence for two distinct agglutinable substances or antigens for the motile members of this group. Also, he studied the microscopic behavior of somatic and flagellar agglutination. In flagellar agglutination the bacilli ceased moving abruptly

and collected gradually into loose clumps with clear spaces between the individual bacilli. Then the clumps gradually became denser. With flagellar agglutination the flagella became coherent, the individuals in the clumps were held together by the coherent flagella, but the bodies of the bacteria did not come into contact with each other. The clumps occasionally showed violent movements. In somatic agglutination small and then large clumps formed, each made up of bacilli which were closely coherent by their surfaces, but unclumped and small clumps of bacilli continued to swim about. This was due to the fact that the flagella on those surfaces which were not touching neighboring bacteria were free and moved vigorously and thereby brought about active translation of single bacteria and also of clumps. The movement of clumps was like that seen in a rough motile culture clumped by salt, a phenomenon often observed in a broth culture of a rough motile strain.

In a critical study of the qualitative receptor analysis advanced by Weil and Felix, Craigie (25) substantiated all the findings concerned with flagellar and somatic agglutination. He stated that heat and ethyl alcohol lysed the flagella, that phenol (agar containing one tenth per cent phenol) did not injure the flagella but merely interfered with the capacity of the organism to develop them, that the unsuitability of formalinized bacterial suspensions for the detection of somatic agglutinins was dependent upon the pre-

sence of flagella fixed to the bacteria. If the suspension was shaken vigorously the flagella were detached and normal somatic agglutination occurred. However, this writer interpreted his findings in a manner different from that of Weil and Felix. Craigie found that solutions of typhoid flagellar substance obtained by lysing flagella (heat or ethyl alcohol) gave precipitin reactions with pure flagellar serums and with somatic agglutinating serums. The substance present in flagella which reacted with pure flagellar agglutination serum was heat stable and resisted a temperature of 100°C. for two hours. The H receptor of Weil was the morphologically intact flagellum attached to the organism and it contained the H antigen. Flagellar lysis, destruction of H antigen, reduced the H antigen to a physical state in which it was not demonstrable by the agglutination reaction but which did not deprive it of in vitro reactivity, nor was the in vivo reactivity necessarily altered. Therefore, he concluded that if the labilotrophic agglutinin of Felix is identical with the flagellar agglutinin, the double receptor hypothesis was of doubtful validity.

With the introduction of prophylactic inoculation, first with typhoid and later with the triple (T.A.B.) vaccine, and its subsequent universal use during and since the World War, grave difficulties arose in the interpretation of the Widal test. It was impossible to determine whether the agglutinins demonstrated were due to active in-

fection or to previous inoculations.

Grattan and Harvey (26) apparently were the first to demonstrate the danger of errors in diagnosis that might arise from an increase in inoculation agglutinins. In India, before the war, they showed that in undoubted cases of Paratyphoid A fever, proven by blood cultures, the patients who had been previously vaccinated against typhoid fever showed a marked increase in their agglutinins for B. typhosus. These results were confirmed in later communications by Grattan and Wood (27) and also by Safford (28).

Dawson (29) proposed the use of a peculiar strain of B. enteritidis Faertner for the differentiation of Typhoid inoculation and typhoid infection agglutinins. The same suggestion was made some time later in Germany by Seiffert (30). Conradi and Bieling suggested the term "anamnestic" for the non-specific response in its relation to previous T.A.B. vaccination.

Felix (31, 32, 33) offered an attractive and simple solution for the problem when he introduced his method of "Qualitative receptor analysis". Briefly, he maintained that in non inoculated individuals suffering from typhoid and paratyphoid infections the differential diagnosis could be made for obvious reasons by means of the large-flaked H agglutination which indicated homologous agglutinins; that small-flaked agglutination occurred in a large number of cases; and that group, or co-agglutinins, belonged to the

small-flaking O type. He also maintained that in inoculated individuals the inoculation agglutinins which persisted in healthy and sick individuals and which reappeared and fluctuated markedly in titer under non specific stimuli of various febrile diseases (anamnestic reaction) were always of the large-flaking H type. No diagnostic value therefore could be ascribed to the presence of large-flaking H agglutinins in cases of enteric infections in previously inoculated patients. Prophylactic inoculation did not produce O agglutinins, but only H agglutinins. In these cases serodiagnosis must rely exclusively upon the presence of small-flaking O agglutinins. The negative result which had to be given in the absence of O agglutinins had the same significance as a negative Widal test in the non inoculated.

In his contentions Felix has been supported by Stuart and Krikorian (34) and by Pijper (35). However, the majority of workers have agreed with Arkwright, Gardner, and Mudd in their opinion that O agglutinins are produced in response to inoculation with the proper strains, that is, those that are fully antigenic.

Since the isolation of B. tularense by McCoy and Chapin (36) this organism has been described by the original discoverers and by all subsequent workers as a small, pleomorphic, non-motile, non-sporulating, Gram negative bacillus.

In Japan tularemia has been studied as an endemic disease by Ohara (37) who called it Yato-Byo. Ohara believed

the organism was a cocco-bacillus. Later studies by Kudo and Kobayashi (38) resulted in the demonstration that these bacilli exhibited motility and possessed a capsule-like envelope. These investigators showed that virulence, pleomorphism, and motility were directly related. The unexpected observed motility caused Kobayashi and Kudo to begin a series of experiments to see if flagella could be demonstrated by staining techniques. After many unsuccessful attempts they finally demonstrated by using Saizawa-Sugawara's modification of the silver impregnation method for staining flagella that the organism possessed a single slender flagellum. The length of the flagellum varied from one half to ten times the length of the bacterial body.

After the demonstration of flagella in B. tularense I decided to see if a serologic relation comparable to the O and H agglutinins in typhoid fever existed in tularemia. An attempt was also made to determine, if possible, by serologic means whether there were any antigenic differences between the various strains of B. tularense that were available in the laboratory. Finally it was decided to see if a Vi antigen analogous to that existing in B. typhosus was present.

Methods and Materials

In the preparation of antigen the organisms were grown on Rhamy's medium and on a medium devised by Foshay for the rapid growth of B. tularense. Cell cultures were

grown for less than 48 hours at 37°C., an interval which yielded excellent growth. In the preparation of H antigen the organisms were washed off with saline containing formaldehyde in a concentration of 1 per cent, left at room temperature for two days, centrifuged once to remove most of the formaldehyde, and resuspended in saline. Since the residual formaldehyde was thought to be sufficient preservative nothing additional was added. The O antigen was prepared by two different methods. The first comprised washing off the organisms with absolute alcohol and letting them stand at room temperature for 24 hours after which the supernatant alcohol was pipetted off and the organisms resuspended in 50 per cent ethyl alcohol. It was quite difficult to obtain a homogeneous suspension of organisms due to the precipitating action of the alcohol. Antigen prepared by this method had to be broken up with a fine bore pipette and the coarse particles cleaned by filtration through a small portion of sterile cotton. These difficulties were obviated by first washing off the organisms with a small amount of sterile saline so that a very dense suspension was formed and by then adding an equal amount of absolute ethyl alcohol. The second method of preparation comprised heating a dense saline suspension of organisms to 85°C. for one hour. Agglutinin titers with heat killed antigen lay midway between those of the formalin killed and alcoholized antigen. An attempt was also made to prepare a pure O

strain by growing the organism on medium containing phenol. The latter resulted in failure since the organism would not grow on media containing phenol in as low a concentration as 1:10,000.

Before performing agglutination tests dense suspensions of O and H antigens were diluted to a turbidity equivalent to 500 p.p.m. of fullers' earth. All macroscopic agglutination tests were performed with the usual serum dilutions prepared to titer from 1:10 by adding 0.5 cc. of the standardized antigen to the various dilutions of serum. The agglutination tubes were shaken, then placed in a water bath at 45°C. overnight and read early the next morning. The criterion for reading agglutination was the naked eye detection of the characteristic clumps with concomitant clearing of the supernatant fluid. Results were recorded in terms of 1, 2, 3 and 4, the latter indicating complete agglutination, that is, perfectly clear supernatant fluid with maximal degree of clumping. The other numbers represent 25 per cent proportions of maximal agglutination.

The serums examined for O and H agglutinins were obtained from normal individuals, from tularemia patients at various stages of the disease, from recovered immunes and from persons who had received B. tularense vaccine prophylactically.

To determine possible antigenic differences among the various strains of B. tularense high titer homologous

immune serums were prepared from rabbits by immunizing each rabbit with one of the 18 available strains. On three alternate days each animal was given an injection of 0.5 cc. of formalinized B. tularense suspension of a turbidity corresponding to 6000 p.p.m. of fullers' earth. All of the rabbits were bled 14 days after the last injection. Agglutination tests with each serum were performed with O and H antigen from the 18 separate strains. Because of the inconclusive results obtained it was decided to absorb the homologous agglutinins from each serum and then perform cross agglutination tests with the remaining heterologous strains. There were only 14 of the 18 serums still available for absorption tests.

In performing agglutinin absorption tests, the growth on slants of Rhamy's medium was washed off with each homologous serum. The serums were then placed in a water bath at 45°C., shaken at intervals, and left for a period of five hours. The serums were then cleared by centrifuging and agglutination tests were performed with the homologous antigen. It was possible in about half the cases to add a calculated amount of antigen and obtain complete absorption immediately, however, if this did not occur the serum was absorbed with its respective antigen until all homologous antibody was removed. Then agglutination tests were performed with the absorbed serum and heterologous antigens.

Innumerable attempts were made to obtain an anti-

serum that would protect or exert curative properties in rodents against minimal lethal doses of B. tularense. Rabbits were immunized in every conceivable way with regard to length of immunization, amount of dosage of killed formalinized vaccine and time interval. All combinations resulted in negative results as far as protective powers were concerned, that is, all animals died with the treated animals living slightly longer than the controls. Serum obtained from goats immunized with living highly virulent B. tularense also failed to protect rodents. Since rodents are so very highly susceptible to tularemia this probably means that we have no suitable test animal since immune goat serum obtained after immunization with living virulent B. tularense seems to exert a more favorable influence on the course of the disease in humans than one obtained by immunization with killed virulent organisms.

Discussion

No attempt was made to study serum from large numbers of normal persons since my experience and that of other workers have shown that agglutinins for B. tularense are never present in such serums. During the past seven years I examined serum from many suspected tularemia patients and also from individuals suffering from other diseases. In no instance was there agglutination with the O and H antigens of B. tularense. Cross agglutination with B. tularense O and H antigens as a non-specific reaction occurred only when

there was a high titer, due to infection with one of the varieties of brucella melitensis.

Table one represents a sample of the agglutinin titer for O and H antigens in patients at various stages of the disease. Agglutinins for B. tularense O or H antigens in agreement with the usual antigenic response were never present prior to the eighth or ninth day of disease. With the production of antibodies agglutinin titers rose progressively to high degrees. Titers for the H agglutinins were always much higher than those for the corresponding O agglutinins and apparently there was a simultaneous agglutinin response. In this respect the antibody response was different from that observed in typhoid fever where there may be only O agglutinins in the early stages of disease, and where H agglutinins appear later. Thus there is slight probability of error in diagnosing tularemia by the use of only an H agglutinating antigen since H agglutinins to a much higher degree than O agglutinins are a constant finding.

For the past nine years prophylactic vaccine has been administered to a large number of people in this area who handle rabbits, some of which are known to be infected with B. tularense. Table 2 shows O and H agglutinin titers for B. tularense in persons recently immunized. Individuals in this group were bled over a period of time ranging from 8 to 44 days after the last injection of prophylactic vaccine. The table shows that H agglutinins for B. tularense were

always present to a higher degree than the O agglutinins. Nearly all of the serums possessed agglutinins for the H antigen in dilutions varying from 1:10 to 1:2560. Three of the 65 serums tested possessed no agglutinating power. nineteen of the 65 serums examined for the presence of agglutinins gave negative results. Serums possessing agglutinins for the O antigen produced agglutination in serum dilutions ranging from 1:10 to 1:160.

In determining whether or not the strains of B. tularense differ antigenically, inconclusive results were obtained. Because of their volume, the tables showing cross agglutination tests are omitted. However, when the serums were absorbed with the homologous antigens, and the homologous antibody completely removed, cross agglutination tests with the heterologous strains showed that agglutinins for heterologous strains were removed along with the homologous. It is evident from results of cross agglutination tests with absorbed serums that there is no appreciable difference in the antigenic composition of the various strains of B. tularense examined.

Thus it is concluded that:

1. Flagellar and somatic antigens are present in B. tularense.
2. The corollary of the above is also true; namely, that flagellar and somatic agglutinins are present in immunized individuals and in tularemia patients.
3. In tularemia the H agglutinins are an invariable finding

and an H agglutinating antigen alone would not give false agglutination results. The O agglutinins are also present but to a much lower degree.

4. There are no apparent antigenic differences in the strains of B. tularense examined.
5. The O agglutinating suspensions prepared by heat and the alcohol method may just be poorer antigens than those prepared by the formalin method.

Morphology

Divergent views exist concerning the morphology of Bacterium tularense. Although capsules were described in McCoy and Chapin's original account of this organism (39), and although flagella were demonstrated on both Asiatic and American strains by Ohara and his associates in 1935 (40), most European language reports on morphology omit all reference to flagella, state that the bacterium is non motile, present inadequate descriptions of its extraordinary pleomorphism, and give scant mention to encapsulation except as an occasional finding in tissues or in tissue smears. Despite an adequate description of involution forms presented by McCoy and Chapin an alternative classification of the species, emphasizing its dissimilarity to B. pestis and based partly upon the absence of flagella and of involution forms in old cultures, has been proposed (41).

Since all previous publications dealing with flagella have been written by Ohara and his associates we have made a thorough review of the Japanese literature. Ota (42) states that Kudo and Kobayashi, working in Ohara's laboratory, first demonstrated a single polar flagellum in 1934, using the silver deposition method of Saisawa and Sugarawa. They also observed capsules. Ota conformed this work. He demonstrated flagella also with Victoria Blue (4 R), Burri's India ink method, and by his own modification of Benian's congo red method. In his experience the methods of Loeffler, Benian,

Zettnow, Inouye, Yokota, and Uyeno either failed entirely or showed few poorly stained flagella.

Ota stained B. tularense and Yato-byo bacteria, also their flagella, in tissues with the Levaditi method. Successful preparations were made from human lymph node and skin, guinea pig spleen, and rabbit liver. Under dark field illumination, "Refractile flagella were demonstrated." With regard to motility, "I found some actively motile, definitely changing their position."

Capsules were well demonstrated by Ota's modification of Benian's method, mercurochrome negative staining, and by Gin's India ink - carbol thionine method. The methods of Johne, Wadsworth, Hiss, Welch, and Friedlander were said to stain them poorly or not at all.

Ohara, Kobayashi, and Kudo (40) used the Saisawa-Sugarawa silver deposition method. They describe the typical B. tularense as a coccus or bacillus with a single polar flagellum. Yato-byo bacteria were said to have longer flagella than American strains of B. tularense. They noted bacterial forms connected by "flagella", also apparent multiple flagella, and instances of three cocci united by "flagella", but assumed that these images were preparational artefacts. Their American strains were 38, Hen, Va, and Col, all derived originally from E. Francis, and sent to Kudo by us.

With regard to motility these workers stated that the bacterium "has a certain active movement", and that the degree

of motility was proportionally related to virulence. They describe its motility as less than that of V. cholerae but greater than that of E. typhi. Addition of a drop of mercuric chloride solution to the bacterial suspension caused motility to disappear slowly, leaving only Brownian movement.

Ohara (43) further mentions the "specific active movement" and states that the organism is monotrichate with a polar flagellum, also a capsule. A privately printed reprint of this complete paper, containing excellent photomicrographs, was presented to members of the Third International Congress of Microbiology. Ohara's statement that the marked difference in severity between tularemia and Yato-byo is not due to inherent differences in virulence between Japanese and American strains, but rather due to the constitution of the Japanese people, should be modified in accordance with the experience related by Ota (42).

We wish to record results of careful and systematic re-examination of the moot points in regard to flagella, motility, encapsulation, and pleomorphism, including the so-called involution forms.

Methods and Materials

Forty-three strains were examined; 21 from our collection and 22 additional ones supplied by Dr. Edward Francis from his collection at the National Institute of Health. The histories of these strains are shown in the table. Attention is directed to the wide range in geographic origin, to diversity of pathologic sources, to an almost complete series of

annual original isolations from 1920 to 1942, and to the periods of cultivation on artificial media which extended from 22 years to 2 days. The virulence range extended from maximal - killing all mice, guinea pigs, and rabbits in 4 to 5 days after parenteral injection of 0.5 cc. of 48 to 72 hour cultures in saline dilution of T-500 x 10^{-9}, to absolute non virulence - no illness in any of 12 mice and 12 guinea pigs after injection of 12 billions of bacteria from a 48 hour culture into each animal.

The methods used to stain flagella were those of Gray, Saisawa-Sugarawa, Casares-Gil, von Ermengem, Leifson, Inouye, Fontana-Tribondeau, and Weiss. For capsule staining we used the methods of Churchman, Welch, Anthony, Wherry, and Hiss; also the flagella stains.

Motility was studied in salt solution hanging drops, and in vaseline-paraffin luted cover slip preparations, by both direct illumination and dark field examination. Our associates, Tamura and Gibby, (50), have devised semi-synthetic and synthetic liquid media in which maximal growth is developed from inocula of approximately 15 organisms to 5 cc. of media. Cultures can be maintained in these media for months without morphologic changes or loss of initial virulence. Observations on morphology and motility were made in luted cover slip preparations of our strains in these media. Some strains had been propagated in liquid media for months; others were sown into liquid and observation prepar-

ations were made daily for eight days. Distinction between living and dead cells was easily made under the dark field. Living cells displayed vigorous intermittent protoplasmic activity; dead bacteria showed no intracellular activity.

Staining technic. Preparation of bacterial suspensions.

Most cultures were grown on solid media for 24 to 72 hours. Physiologic salt solution proved better than distilled water as a suspending medium. Satisfactory suspensions were made in either of two ways.

1. A small amount of surface growth was removed with a loop and very gently shaken into a tube of salt solution. The tube was incubated at 37° for 1 to 2 hours until a uniform suspension resulted. Occasional gentle rotation accelerated this process but shaking broke off most "flagella". The cohesive nature of the growth made it difficult to dislodge it from the loop.

2. One or 2 cc. of salt solution was pipetted into the end of a culture slant, the tube incubated until there was visible turbidity, then some of this suspension was transferred to another tube of salt solution. This was the better method.

These suspensions were good for "flagella" demonstration for at least 3 days. Capsules were best shown during the first 6 hours. Preparations were also made from growth in liquid media.

Thin air dried films were stained on both slides and

cover slips. Preheating slides in a gas flame gave preparations with the clearest backgrounds. Although the Gray stain must be mixed for each day, we found it left less background deposit if it was allowed to stand one hour before using. Beyond this, the technic was simply that always necessary for success with special staining methods.- scrupulously clean glassware, experience with the methods, and patience.

Demonstration of capsules

Capsules were best demonstrated by either the Gray or Saisawa-Sugarawa flagella stains; less well by the Casares-Gil stain. We were unable to demonstrate them with the Churchman, Welch, Wherry, Anthony, or Hiss staining methods, though modifications of each were tried.

Well defined capsules were demonstrated on every strain. The coccoid form, whether of minute, small, medium, or giant size, was usually encapsulated. Bacillary forms with capsules were rarely seen. When cocci and bacilli appeared in chain formation it was usual to find only the cocci encapsulated. Giant cocci often showed thick capsular membranes. Frequently two minute circular clear areas, or indentations, were noted within the thick membrane walls, usually almost exactly 120° of arc apart. Wehn such forms possessed "flagella" these structures seemed to arise from these circular areas.

Capsules were formed by avirulent as well as by virulent strains; by recently isolated cultures as well as by

old cultures propagated on media for more than 20 years. The capsule is an integral part of the structure of the organism, with no relation to virulence or invasiveness. Sohkey showed very convincingly that the same is true of the plague bacillus (44). In this respect B. tularense and B. pestis are alike, both differing from many common pathogenic bacteria.

Involution forms.

Classical involution forms were observed in about half of the strains. Although typical examples were seen and photographed in an avirulent strain propagated only on media for 22 years, they occurred more frequently in virulent strains, and most frequently in recently isolated cultures. Dumb-bell, bean shaped, spermatozoon-like, and many bizarre forms were seen, as well as L-shaped and irregularly knobby globoid forms. Long filamentous forms like those produced by the plague bacillus were not observed, but many shorter and bizarre filamented forms were seen, some of which are reproduced.

The appearance of flagella in stained preparations.

Very fine filamentous structures identical with those the Japanese students call flagella were observed in every strain. They were best demonstrated by the Gray, Casares-Gil, and Saisawa-Sugarawa staining methods. Excellent preparations were made by each method. We were unable to demonstrate these structures with the staining methods of von Ermengem, Leifson, Inouye, or Weiss. Prolonged staining with

Wright's stain or freshly prepared hemotoxylin occasionally demonstrated them but poorly. The pale colors, and the minute size due to absence of previous mordanting, made such preparations poor subjects for photography. One plate was obtained from which measurements could be made.

In the unmordanted state the usual size coccoid form has a diameter of 0.3 u to 0.5 u. Bacillary forms range from 0.3 by 0.9 u to 0.5 by 2.5 u. The form known to us as the minute coccus was not seen in unmordanted preparations. After heavy mordanting these forms have a diameter not greater than 0.2 u, and cannot be distinguished from precipitated stain unless they possess a "flagellum". Giant cocci have diameters as large as 3-5 or 4.0 u. Length of "flagellum" varies greatly, from 0.5 u to 8.0 u or longer. A few specimens stained without previous mordanting showed "flagella" of uniform thickness, but really so thin that they were discernible with difficulty under critical illumination. They appeared to be not greater than 0.05 u in thickness.

Although Ohara states that the longer "flagellum" of yato-byo bacteria differentiates them from B. tularense, we were unable to find any significant differences among our 43 strains.

These flagella-like structures were seen most frequently on coccoid forms, regardless of size; less frequently on bacillary forms. Many showed bulbous tips at the free end. Others frequently showed a normal coccus or bacillus at

the end, giving the appearance of two bacteria united by a fine filament. Other fine filamentous structures united 3, 4,5, or more than 20 bacterial bodies of either coccoid or bacillary shapes. The appearance of multiple flagella was noted also. At first we thought all such images were preparational artefacts and, indeed, by judicious variations in methods of preparation, especially in depth of mordanting and staining, most such images from a given suspension could be resolved into apparently monotrichate bacteria lying very near free "flagella", the free end of another "flagellum", and so forth. Although light mordanting revealed many such images as artefacts it did not dispose of the matter of various bacterial forms united by fine filaments. Every strain continued to show such united forms under most critically controlled variations in technic.

Differentiation between flagella-sized filaments and capsular streamers due to traumatic rupture of capsules was easily made. Filaments stained evenly and deeply, with sides parallel throughout their length. Capsular streamers stained lightly, often unevenly, and tapered from a broad base near the soma to fine single or multiple distal tips.

When the so-called flagella appeared on encapsulated bacteria they sometimes seemed to arise from the soma and penetrate through the capsular membrane. Others seemed to arise from the capsular membranes. Similar appearances of B. subtilis were noted and discussed by Churchman (45). We believe this

disparateness is due to variations in staining technic. From a suspension of B. tularense, or of Ps. aeruginosa, we made preparations which showed first one appearance, then the other. We have occasionally seen both forms on the same slide, the flagellum not visible through the capsule in lightly stained areas, but well shown within the capsule in more heavily stained areas; best seen with Ps. aeruginosa.

In some encapsulated B. tularense the "flagellum" was very clearly seen coiled within the intact capsule. This is shown in some of the photomicrographs. In the giant cocci the intracapsular location of the filament was exceptionally well shown, where they commonly heavily accentuated about one third of the perimeter of the capsule. If capsules had been broken by trauma these filamentous structures could frequently be seen partially uncoiled from the original position, and easily differentiated from the associated capsular streamers. One photomicrograph shows this arrangement very clearly.

The coccoid form is the one most frequently seen, and most "flagella" and most capsules are seen on this form. It is the only form in which "flagella" were observed within capsules. One might argue that, if the filaments observed are true flagella the almost universal failure of skilled observers to see motility of B. tularense might result in part from the frequent intracapsular location of flagella, especially since more than 3 hours are required for capsules to disintegrate in salt solution. This hypothesis implies that proper or

suitable conditions for observation of motility might never have been secured, an hypothesis which was readily made untenable by our study of living cultures in extremely favorable liquid media by dark field illumination.

Observations on living cultures under dark field illumination.

Morphology.

All of the usual forms seen in stained preparations were readily observed under the dark field. Minute coccoid bodies, cocci of usual size, larger, and giant cocci, bipolar forms, short and long bacillary forms, and true filaments of bacillary transverse dimension were noted. All sizes of cocci and bacilli appeared singly, in diploform, and in short chains, sometimes in chain formation with filaments of various lengths. Involution forms were also seen. All strains had the same range of pleomorphism.

The "flagella" described and photographed by Ohara and his associates were readily seen. Their appearance in dark field preparations was identical with that in fixed preparations stained by the Japanese, in similar preparations made by us, and in the photomicrographs published by Ohara as well as in our own. However, all such "flagella" were absolutely non motile, whether observed in water, salt solution, or liquid media. We watched many living preparations all day for many days, making a fresh one for a single culture every day throughout its period of multiplication. We saw thousands of these flagella-sized filamentous structures attached to all observed

forms of the organism except certain involution forms, but they were invariably non motile. The bacteria multiplied well in the liquid medium, and there was never any doubt concerning viability of cells observed. Suspended dead cells, with or without "flagella", were mere shadow outlines of living ones, but all, living or dead, showed only Brownian movement. The easiest way to locate a cell with a long "flagellum" was to look for one which, for its diameter, had less Brownian movement than would be expected. The longer the filament the more it restricted Brownian movement in rate and amplitude. If there was also a bacterium at the other end of the filament it always acted as a drag anchor, permitting almost no Brownian movement. Multiple "flagellated" living forms were seen, confirming our findings in stained preparations.

In living cultures filaments of flagella size were seldom attached to only one organism. In stained preparations this appearance was very frequently noted, simulating true flagella, but it seems almost certain that this form is usually an artefact caused by trauma incident to the preparation of dried films. In cultures the great majority of fine filaments connected two or more organisms of any of the forms noted.

We can offer a possible explanation for the "peculiar motion" so frequently mentioned by Ohara and his coworkers. All bacillary forms and all true filamentous forms exhibit active flexional movements. These squirmings rarely accomplish any motion of translation, but occasionally an especially

vigorous bend will effect a change in position of a micron or two. We frequently left such forms in a field for an hour, to return and find the bacillus or filament not more than one half field diameter from the point at which we left it. Ohara writes of thin and thick flagella. From our observations we believe all "thick flagella" to be slender bacilli or true filaments. When a long bacillus, or true filament, had a coccoid form at one end, noted as the "drum-stick" forms in our photomicrographs, the flexional movements of the rod-shaped section gave the appearance of a thick flagellum. We watched many such forms, and the greatest rate of movement of translation recorded was of the order of 15 u per hour. We can confirm the constant presence of a capsule, flagella-sized filamentous structures, true filaments of bacillary transverse diameter, involution forms, and extraordinary pleomorphism, but not motility.

We kept a daily free-hand pictorial index of our observations at different stages of growth of the various strains. We could not detect any special or predominant morphologic phase at any given time during the first 8 days of growth. Even with very small inocula it seemed that as soon as there were enough bacteria present to show 15 to 20 to the oil immersion dark field, prolonged search would reveal at least one example of every form yet observed.

The figure shows our drawings from dark field observations of living cultures of some of the strains at various stages of growth. It must not be inferred from these drawings

that the forms shown were present in every field. At least one example of every form drawn was observed in every strain, but the filamented and other bizarre forms represent only a small fraction of the total population. The most numerous forms seen during any of the first 8 days of cultivation were always the coccoid and bacillary forms. Many of these were not filamented.

Observations of the mode of reproduction of B. tularense.

We never observed reproduction of coccoid and bacillary forms by the usual method of binary fission. On the contrary, B. tularense appears to possess at least two alternative forms of reproduction.

The giant coccoid forms, usually more abundant in young vigorous cultures, were seen to reproduce by budding. The appearance of budding forms was identical with that seen in stained smears except that whereas the smears showed buds on cocci of various sizes we observed the formation of buds best on the giant cocci. Buds appeared from the capsular membranes at the sites of the clear circular areas previously described. These buds developed into usual sized coccoid forms. Chains of cocci developed at a single budding site. Such chains may or may not show filamentous linkage between members. Chains seldom exceeded a total of four members unless they were of the filamented, "beaded" type. We saw terminal non-filamented chained cocci break off singly and in pairs. Thereafter the same giant coccus sprouted two more buds at

new sites about 120° of arc apart and resumed formation of non-filamented coccoid forms. There was considerable protoplasmic commotion at the budding site, and six or seven rapid out-pouching and retracting movements were made through the capsular membrane before the bud finally stayed outside. This process was repeated for each additional coccus as it was added to the chain.

We saw one giant coccus add a minute solid spherical coccus, by extrusion, to a long filamented chain which already consisted of two usual sized cocci, a long bacillus, and a long terminal non motile filament. Here again it required 5 or 6 intermittent, vigorous out-pouching or extrusive movements through the capsular membrane at the site of junction with the fine filament, each out-thrust apparently surrounding and enclosing the proximal end of the filament, before the new coccus was delivered to a permanent place in the filamented chain. At no time did we observe formation of the fine filaments.

In addition to the two alternative modes of reproduction observed, by budding and by pullulation along filaments, many of the extraordinary morphologic forms seen in our photomicrographs and drawings bear a striking resemblance to certain growth phases of organisms of the pleuro-pneumonia group as they are depicted in studies by Ledingham (46), Klieneberger (47), Turner (48), and Sabin (49). Many outstanding differences were also noted. The coccoid forms of B. tularense,

for example, were always spherical or ovoid, not discoid. Branching filamentous structures were never seen in living preparations, and the rare appearance of branching in stained films is almost certainly artefact. Turner (48) mentions the amazing rapidity with which certain larger forms of pleuropneumonia organisms change their shape reversibly. He cited the example of a long bacillary form, or filament, with double walled contour under the dark field, which rapidly assumed the appearance of a short chain of bacillary forms, then reverted to the former appearance. We have observed the same phenomenon with true filamentous forms of B. tularense, but only when the structure became partly adherent to the cover slip or the slide, never when the organism was in suspension between the glass surfaces. We have not studied pleuropneumonia organisms, and we hesitate to offer comment other than admiration for Turner's magnificent work. We suggest that the example of rapid reversible morphologic change cited was an artefact due to adherence to a glass surface, especially since Turner rolled his cover slips down against the slides until Newtonian rings appeared. This comment cannot apply to other rapid morphologic changes observed by Turner, such as the growth of germinal tubes.

Observations on supra-vitally stained living organisms.

It is well recognized that artefacts are frequently produced in preparations that have been fixed and stained.

Our photographic equipment did not permit direct photography of living organisms under dark field illumination, the form of study producing the fewest artefacts. Consequently, we devised methods to stain living organisms in gelatin hydrolyzate cultures and in saline suspensions of growth removed from the usual solid media.

Luted cover slip preparations were made as usual, with the addition of an amount of dye equal to the amount of bacterial suspension. Most dyes were used in saturated saline solution. Of 29 dyes studied in this manner, examining each preparation both in bright field and in dark field, Nile blue sulphate, Hoffman violet, malachite green, methylrosaniline, safranine O, Janus green B, and Bismark brown, proved useful. The nuclear dyes stained all chromatin material, including buds, minute granules within filaments, and these minute granules in other locations. Bismark brown stained all of these structures, and also revealed the filaments and the limiting membranes of all forms seen. Therefore, this dye permitted study, and photographic records, of both external and internal morphologic structures.

Coccoid forms were often not coccoid in shape, especially the smaller, younger ones. They were ring-shaped, discoidal, navicular, or shaped like a rounded up segment of an orange. The chromatin or nuclear material was always peripherally located, not centrally located as in most bacteria. Filaments and filamentous connections were well seen. Larger coccoid forms were globoid in shape. The so-called bacillary

forms were not bacillary in shape, with the two short diameters approximately equal. They were ribbon-like, or lath-shaped, with one short diameter very much smaller than the other, very unlike most bacteria. Thick filaments could be seen occasionally within globoid forms, showing that this appearance, noted in stained fixed preparations, was not an artefact. Long chains of budded ring forms were seen, with occasional side budding from one or more elements forming the appearance of branching. Careful examination showed that this was always false branching due to differential budding. The same held true for the apparent branching observed in bacillary forms or in chains of these forms, or in mixed element chains composed of both ring and bacillary forms.

Sessile and filamentous budding were clearly observed. The chief mode of reproduction appeared to be by various methods of budding, and by the formation of what appeared to be minimal reproductive units. These minute units formed at the ends of fine filaments, at various locations along the courses of filaments, and in meridional bands around the large globoid forms. In some instances a single filament branched into a cluster of fine terminal filaments, each with a minute minimal reproductive unit at its tip. Single minute units were formed filamentously by ring forms, globoid forms, and bacillary forms.

The supra-vitally stained preparations demonstrated

clearly the nature of the forms that looked like involution forms in fixed and stained smears. These were large, globoid forms, often with budded chains of small rings attached to them, simple budding of globoid forms from other large globoid forms, "L" shaped budding from bacillary forms, and complex mixtures of these various forms united either by short filaments or directly by sessile budding.

Evidence was obtained that the small buds and the minimal reproductive units were of different protoplasmic constitution from the nuclear chromatin material. With malachite green all nuclear chromatin stained green, but all buds and minimal reproductive units stained brick red. With Hoffman violet, nuclear material stained deep violet, whereas buds and units stained a light rose-lilac shade. These differences indicate chemical differences in constitution and, most probably, differences in antigenic specificity.

Since all organisms now classified in the pleuropneumonia group are characterized morphologically by ring forms, globules, filaments of various sizes, the formation of minimal reproductive units, and multiple modes of reproduction, it is apparent from these studies that the organism we have long called Bacterium tularense bears close morphologic resemblance to members of this group. Indeed, it meets all morphological requirements for classification among the pleuropneumonia organisms. It differs from all known pleuropneumonia species in that micro colonies of 10 to 600 mu are not formed on serum agar. Also, growth will not occur

in simple 30% serum broths or ascitic fluid broths, standard media for cultivating most pleuropneumonia species. A further striking difference between Bacterium tularense and all known pleuropneumonia organisms is exhibited by their respective host relationships. Hitherto, a prominent feature of pleuropneumonia species has been their strict host relationships. Pleuropneumonia bovis infects only cattle, no other host. Asterococcus canis infects only canines. Anulomyces agalaxiae infects only sheep. So also with the strains isolated from rats, mice and other rodents. On the other hand, Bacterium tularense has a veritable catholicity of taste with regard to mammalian, insect, arthropod, amphibian, marsupial, and reptilian hosts. There are at least 40 known natural animal hosts and approximately 50 hosts and natural vectors among insects and arthropods.

Significance of these Studies

During the past 30 years much information has been gradually acquired about tularemia or Bacterium tularense which has cast doubt upon the correctness or propriety of the present classification of the causative agent among the Pasteurella group of bacteria. With regard to the organism itself, certain so-called unique features have been recognized. It has obligate growth requirement for the amino acid, cystine. It will not grow at all in usual liquid media, even if cystine is supplied. The organism perishes in the water of condensation from the most favorable solid medium devised for it. It

is an obligate aerobe, demanding atmospheric oxygen, and has no facultative anaerobic capacity whatever. Indeed, better growth is obtained in all media if an atmosphere of 20 to 30% of oxygen is supplied. It stains poorly with all ordinary dyes. In animal tissues it stains with difficulty, and it is found in intracellular locations in various types of cells in rodent tissues. It has very seldom been seen in human tissues, although transmission experiments indicate they must be extremely numerous in such tissues. It penetrates normal, unbroken skin, of man and animals, with ease and rapidity. The disease it produces in rodents is almost invariably a fatal septicemic infection. In man septicemia seldom occurs. Although all men are equally and highly susceptible to infection, most acquire rapidly a high degree of resistance soon after infection. Mortality in man is about 6 per cent. After recovery from attack man is absolutely refractory to subsequent infection, a degree of protective immunity not noted for bacterial infections. Serum agglutinins, once acquired by infection, persist for the remainder of life of the recovered patient. After recovery from the pneumonic form of the disease, the sputum may contain organisms highly fatal to all rodents for many weeks after the patient is fever-free. In insects and arthropods, the organism parasitizes epithelial cells. In at least three species of ticks, the virulent organism is hereditarily transmitted by the infected female to her eggs, to the larvae, to each of two

nymphal instars, and finally to the adult of the next generation. Although very difficult to cultivate in any liquid medium prior to the cited work of Tamura and Gibby, the organism survives for many months in a virulent form in water of pooled and flowing streams, and in the mud of these streams.

All the above attributes, well authenticated and established, are incompatible with the behavior of any bacterium known to us. The recognition of the organism for what it is, offers an adequate or partial explanation for all of these so-called unique features, and will direct research to solve still other unknown problems concerning tularemia into more profitable channels. As long as the fixed idea that the organism was a bacterium dominated investigative thought, few notable advances were made toward solution of many unique and perplexing problems. The present studies clear the way for application of different and more useful methods of investigation.

Summary

Bacterium tularense is an extraordinarily pleomorphic organism. It is probably not a bacterium. It is extremely doubtful if its present classification under the order Eubacteriales is appropriate. Although it is unlike pleuropneumonia organisms in certain stated respects, its production of ring forms, globoid forms, filaments, minimal reproductive units,

as well as its possession of multiple modes of reproduction, indicate it is more closely related biologically to the pleuropneumoniagroup than to any other group of microorganisms known at present. It seems best to postpone attempts at biologic classification until the pleuropneumonia group, and Streptobacillus moniliformis (Actinomyces muris), are satisfactorily classified.

Bacterium tularense is not a flagellated organism, and it is devoid of motility. None of the usual terms used to describe bacteria are appropriate for its description. Thus, the long continued discussions about whether or not it is flagellated, encapsulated, or forms involution forms, are no longer even a matter of academic interest.

Although the alleged flagella have been shown to be inert filaments, the serologic studies demonstrated that the filaments, or other extra-somatic structures, are chemically different from somatic protoplasm and have different serologic specificity. For practical purposes, and until knowledge is extended, these serologic differences may be regarded as analogous to those of O and H agglutinins and agglutinogens of motile bacteria. Consequently the "O" and "H" antigens of Bacterium tularense, and their respective antibodies, remain practically useful in differentiating infected individuals from immunes and from vaccinated persons.

It was not possible to demonstrate morphologic differences between avirulent strains and strains of maximal

virulence. The envelope or capsular membrane is an integral part of the basic structure of the organism, with no relationship to invasiveness or virulence. In this respect Bacterium tularense differs from most pathogenic bacteria, although the plague bacillus is another notable exception.

The reciprocal antibody absorption studies carried out on 14 American strains, including a totally avirulent one, indicated that all 14 strains were antigenically identical. Efforts to disclose the presence of a deep somatic antigen that might be associated with virulence, analogous to the Vi antigen of typhoid bacilli, were unsuccessful. The nature and location of the factor, or factors, that are responsible for virulence or non virulence remain completely unknown.

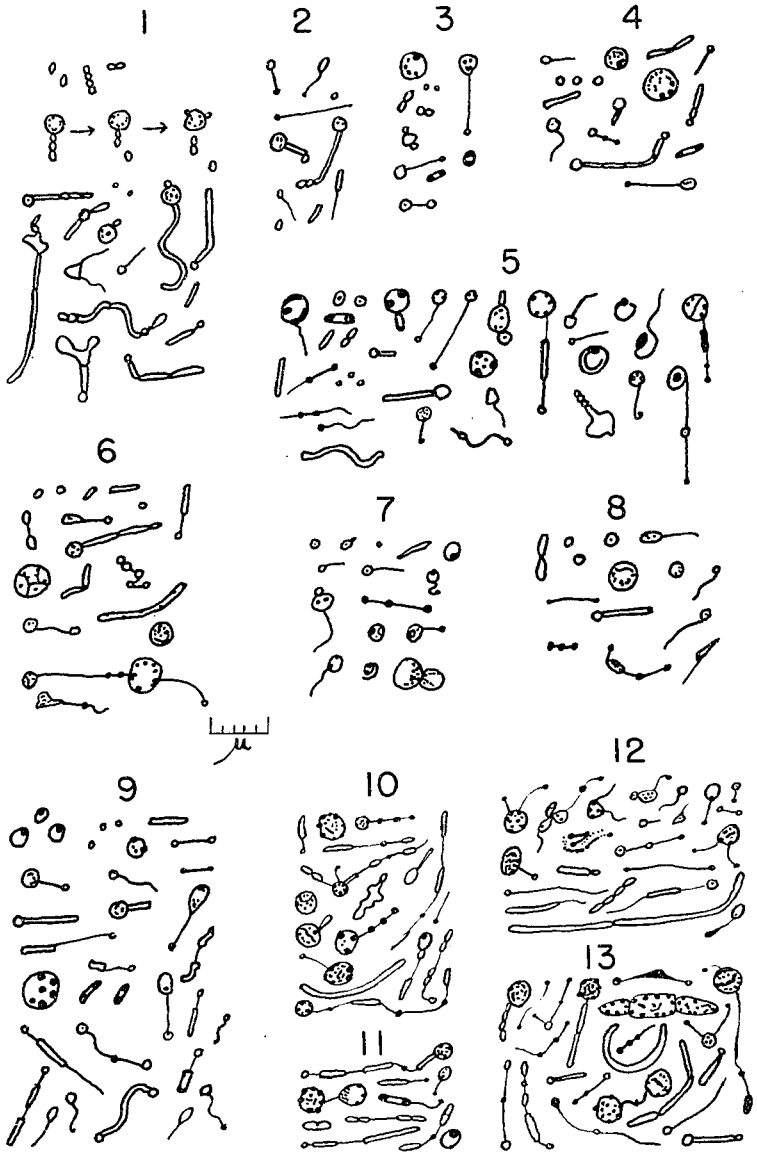

Fig. 1.

Examples of diverse morphologic varieties of B. tularense drawn from observation of living cultures in liquid media under dark field illumination. The preparation from which Fig. 9 was drawn was made by suspending in salt solution a small amount of growth removed from a blood-cystine-agar slant; included to show that the same diverse forms are produced by cultivation on the standard solid medium.

Fig. 1. Strain Memp. 19 Hrs.
Thirty to 40 minutes elapsed between the successive stages indicated by the arrows, reading from left to right. Note reproduction by budding. Coccoid forms are ovoid.

Fig. 2. Strain Memp. 44 Hrs.

Fig. 3. Strain Memp. 90 Hrs.

Fig. 4. Strain Memp. 5 days. Coccoid forms have become spherical.

Fig. 5. Strain Memp. 6 days.
Note the filament coiled within the capsule of one giant coccus.

Fig. 6. Strain Jap. 24 Hrs.
Filamented zooglea-like mass at bottom.

Fig. 7. Strain Ohara.48 Hrs.

Fig. 8. Strain Ohara 72 Hrs.
At bottom, filaments sprouting from an illy defined zoogleal mass.

Fig. 9. Strain Schu. in salt solution. 72 Hr. culture.
Signet ring forms, at upper left, were very common in all strains.

Fig. 10. Strain Pack 48 Hrs.
Reproduction by pullulation along filaments was observed in forms shown at the bottom.

Fig. 11. Strain Pack. 72 Hrs.
Division by fission was well observed, indicated near bottom.

Fig. 12. Strain Ri. 48 Hrs.
Note multiple filamentation at upper left. Just below is a zooglea-like mass showing apparent fragmentation of filaments.

Fig. 13. Strain Russ. 48 Hrs.

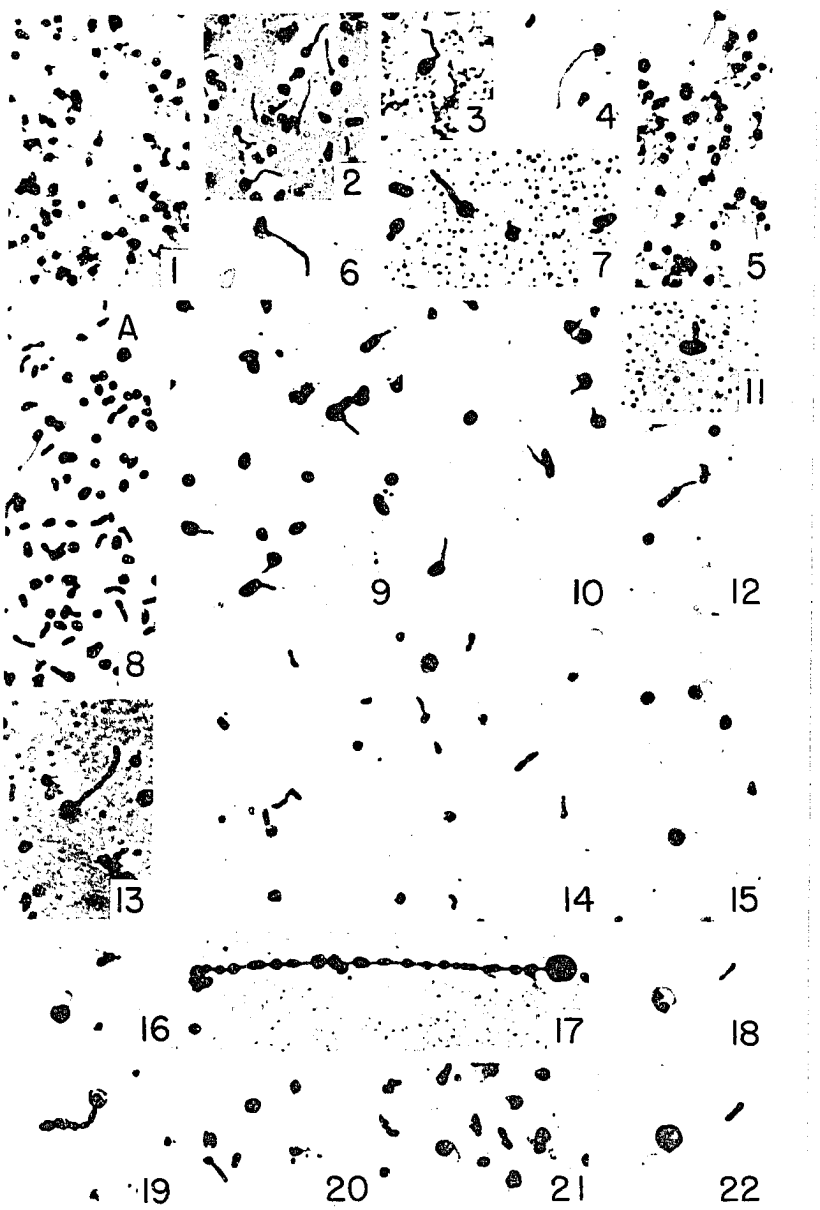

Fig. 2.

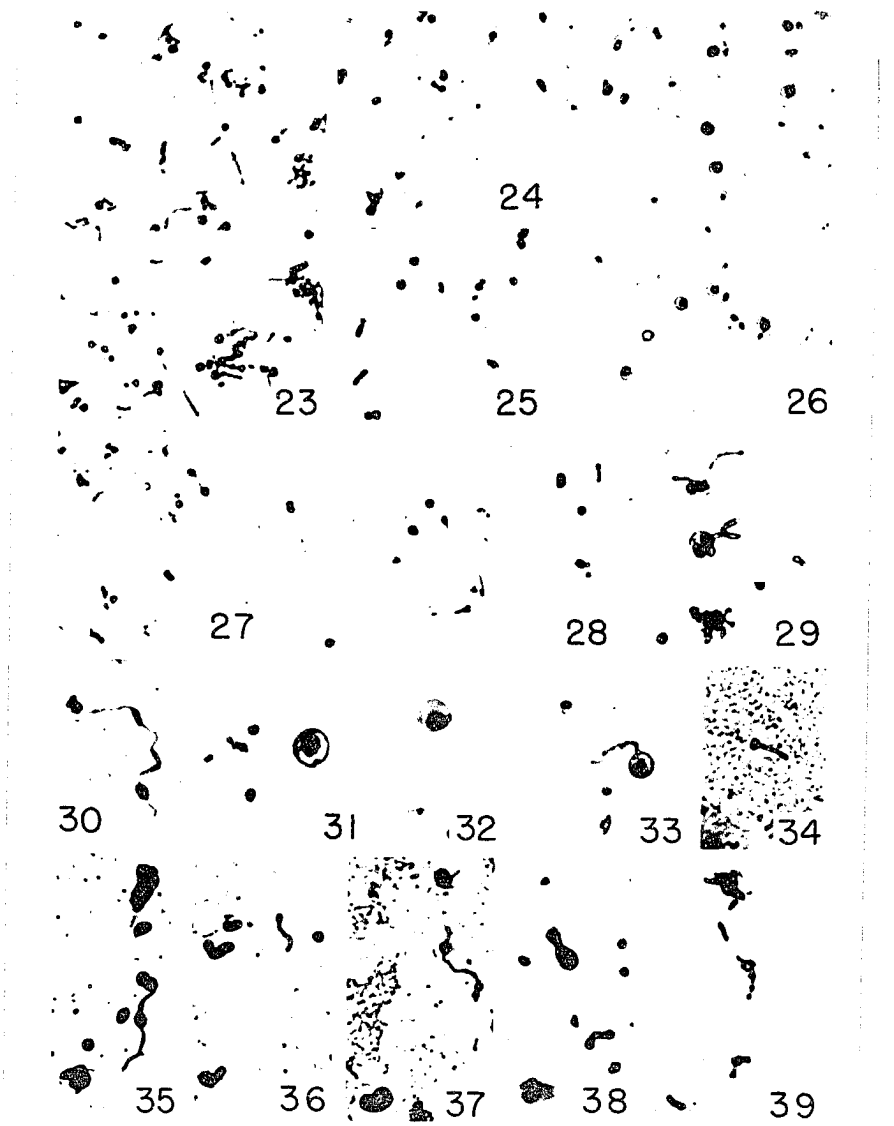

Fig. 3.

Figures 2. and 3.

Photomicrographs of fixed preparations of Bacterium tularense, stained by the methods of Gray, Casares-Gil, and Saisawa-Sugarawa. All taken at the same initial magnification of 2250 diameters. Reproduced without reduction.

1. Virulent strain Chr. Saisawa-Sugarawa stain. Usual coccoid forms. Note how closely the filaments resemble flagella; also the small budding form in center near top.

2. Virulent strain Col. Saisawa-Sugarawa stain. Slide prepared by Kobayashi in 1935. Our photomicrograph. Note the minute coccus with a filament.

3. Avirulent strain 38. Gray stain. This strain has been propagated only on media since 1920.

4. Virulent strain Schu. Gray stain. Note the two minute areas of condensation near the middle of the filament.

5. Virulent strain H. D. Casares-Gil stain. Filamented cocci, the filaments closely resembling flagella. The apparent branching near the center is probably artefact.

6. Avirulent strain 38. Saisawa-Sugarawa stain. Note filament extending through capsule.

7. Virulent strain Schu. Gray stain. Short and long filaments. Note budding form at left.

8. Same. Filament connects minute and usual sized cocci. Note capsules on many cocci, "drum-stick" form at bottom. At A a filament is seen coiled within an intact capsule.

9. Same. Flagella-like filaments on both coccoid and bacillary forms.

10. Same. Filament attached at acute angle to long side of a bacillus. Note thickened area near middle of filament.

11. Same. Thick filament attached perpendicularly to long side of a bacillus.

12. Strain Schu. Casares-Gil stain. Bacillus with concave sides and short flagellum-like filament.

13. Same strain. Gray stain, heavily mordanted. Thick filament attached to an encapsulated coccus.

14. Same, lightly mordanted. Encapsulated small and large cocci. Note the "drum-stick" forms, the ones which may simulate true motility in living preparations.

15. Same. False "flagella" produced by streamers from ruptured capsules. At bottom, part of the filament can be seen within the capsule, opposite the site of rupture.

16. Same. Encapsulated coccus with short filament having a minute coccus at its tip.

17. Strain Max. Casares-Gil stain. Long "beaded" chain with filamentous linkage, probably formed by budding from the encapsulated giant coccus.

18. Strain Schu. Gray stain. Focussed to show a segmented capsule containing 2 coccoid forms. The filament of the upper coccus is within the capsule.

19. Same. Non-filamented chain formation. Only the coccoid forms are encapsulated. In living cultures such chains were seen to form by budding from the large cocci.

20. Same. Encapsulated small coccus with filament coiled within the capsule. "Drum stick" form at lower left.

21. Same. Encapsulated cocci with ruptured capsules, showing filaments partially uncoiled from normal position within capsules. Note how readily filaments are distinguished from capsular streamers.

22. Same coccus as Fig. 18. Focussed deeper to show filament of the lower small coccus protruding outside the capsule.

23. Strain Die. Saisawa-Sugarawa stain. Note frequent filamentous linkage of various forms, and the filamented triangular zooglea-like mass at lower left.

24. Strain Ri. Saisawa-Sugarawa stain. Three minute coccoid forms connected by filaments.

25. Same. Note unusual length of flagellum-like filament.

26. Avirulent strain 38. Saisawa-Sugarawa stain. Note capsule formation by this old laboratory strain.

27. Strain Ri. Saisawa-Sugarawa stain. Filamentous linkage of small and minute coccoid forms.

28. Same. Filaments connecting various forms. Bipolar bacillary form at upper right.

29. Strain Memp. Fontana Stain (Tribondeau modification.) Upper shows twin coccoid forms, each sprouting fine filaments containing minimal reproductive units. Middle shows a medium sized globoid form with radially located sessile and filamentous buds. Lower shows a globoid body with a thick wall enclosing two units of usual coccoid size, the whole budding from a long bacillary form.

30. Strain Max. Casares-Gil stain. Filamented chain of cocci and bacilli.

31. Strain Schu. Gray stain. Giant coccus with thick capsular membrane. Note indentations in membrane wall.

32. Same. Giant coccus with delicate capsular membrane, from the same slide as Fig. 31.

33. Same. Large coccus, encapsulated and filamented. Note circular areas in capsular membrane, one at point of attachment of filament. Part of an intracapsular filament is visible in the upper small coccus.

34. Strain Max. Casares-Gil stain, heavily mordanted. Typical flagella-like appearance.

35. Virulent strain Schu. Gray stain. Globoid involution form and filamented cocci in chain.

36. Avirulent strain 38. Saisawa-Sugarawa. Involution forms.

37. Same. Globoid involution form and filamented chain of cocci.

38. Strain 38. Gray stain. Involution forms.

39. Strain Memp. Fontana Stain. Shows development of various forms by budding, and by pullulation of fine filaments.

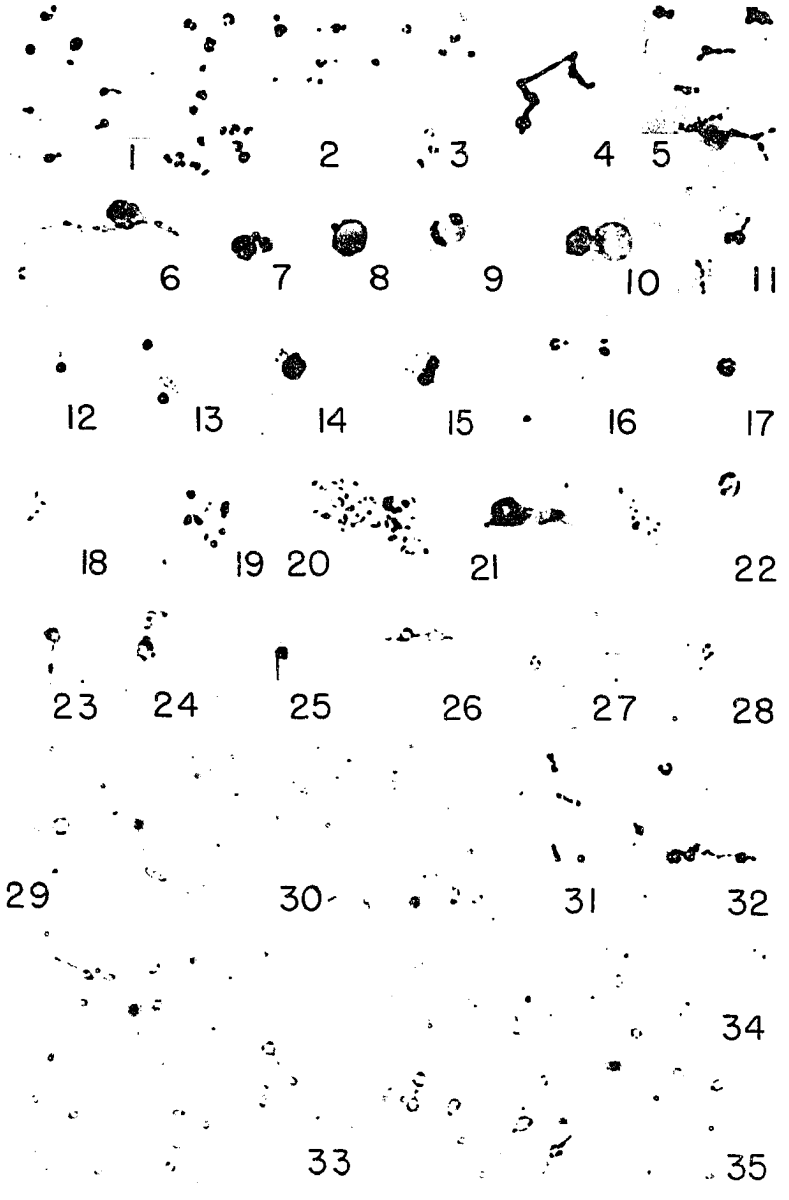

Fig. 4.

Figure 4.

Photomicrographs of Bacterium tularense stained supravitally in luted cover slip preparations. Reduced from magnification of 2250 diameters.

1. Sessile and filamentous budding. Living cultures, Memp and Chr strains, stained by aqueous methylrosaniline or Hoffman violet.

2. Signet ring or coccoid forms, some showing sessile or filamentous budding. Russ strain stained with Hoffman violet in saline solution.

3. Ring forms. Memp strain stained with Hoffman violet in gelatin hydrolyzate medium.

4. Bizarre chain formation; coccoid and bacillary forms. 38 strain stained in saline suspension with Hoffman violet.

5. From top downwards: Multiple budding of coccoid forms. Russ strain stained in saline suspension with Hoffman violet. Branching due to budding. Memp strain stained in liquid medium with Safranin O. Bipolar staining bacillary form. Memp strain, Safranin O. Filamentously connected bacillary and coccoid forms, showing branching. 38 strain stained with Bismark brown.

6. Mycelial-like chain of ring and bacillary forms giving rise to (or budded from) a large globoid form. The globule contains 2 bodies of usual coccoid dimensions. Memp strain in culture. Bismark brown.

7. Consecutive sessile budding from a globule. Russ strain in saline. Hoffman violet.

8. Globule with sessile bud. 38 strain in saline. Bismark brown.

9. Globule with peripherally distributed chromatin. 38 strain in saline. Hoffman violet.

10. Large globule with globular bud. Note the 3 peripheral dots, budding sites, on the mother globule. The daughter globule contains 2 bodies of usual ring or coccoid dimensions. Schu strain in saline. Bismark brown.

11. Twinned ring forms, one filamented; the filament containing 2 minute granules. Below are 4 similar granules filamentously connected. 38 strain in saline. Bismark brown.

12. Coccoid form with extremely slender filament with 2 minimal reproductive units at distal end. Memp strain in culture. Hoffman violet.

13. Coccoid form with a single multibranched filament. Note the (M.R.U.) minimal reproductive units at termini of fine filaments. Russ strain in saline. Safranin O.

14. A 2u globule photographed slightly out of focus to show part of a meridional budding fringe. Russ strain in saline. Hoffman violet.

15. Peripheral filamentous budding from twin globules. Note the M.R.U. along the filaments. Russ strain in saline. Hoffman violet.

16. Signet ring form, filamentous bud on coccoid form and a fine filament on a small coccoid form. Memp strain in culture. Hoffman violet.

17. Ring form with peripheral granules. Schu strain in culture. Hoffman violet.

18. Filamentously connected group of M.R.U. Two show developmental enlargement toward ring forms. Russ strain in saline. Hoffman violet.

19. Filamented and filamentously connected ring forms and M.R.U. Russ strain in saline. Hoffman violet.

20. Clump of developing M.R.U. Some have reached usual ring form size. Some are filamentously connected; others are embedded in a capsule-like substance. Memp strain in culture. Hoffman violet.

21. Ruptured large globule showing parachute-like arrangement of delicate filaments with M.R.U. along their lengths and at their termini. Memp strain in culture. Hoffman violet.

22. Oval globoid form with peripherally distributed chromatin. Russ strain in saline. Hoffman violet.

23. Filamented ring form with M.R.U. in filament. 38 strain in saline. Bismark brown.

24. Globoid form with peripherally located chromatin and a small sessile bud. Above, twinned rings, one with a delicate filament and a M.R.U. at its tip.

25. Filamented encapsulated coccoid form. Memp strain in culture. Bismark brown.

26. Filamentous linkage of well-stained living ring form and unevenly stained dying or dead ring. Note the 2 filaments sprouting from the left side of the healthy ring form. The minute one has a M.R.U. at its tip. Russ strain in saline. Bismark brown.

27. Coccoid ring with peripherally located chromatin dots and a long slender filament with terminal M.R.U. Memp strain in culture. Bismark brown.

28. Ring form showing M.R.U. in its filament. Memp strain in saline. Bismark brown.

29. Globoid form with peripheral chromatin granules and a delicate filament with a minute globule at its tip. Note also the so-called bacillary forms. Memp strain in culture. Bismark brown.

30. Bacillary, ring, and globoid forms. The latter filamented with M.R.U. along the filament. Memp strain in culture. Bismark brown.

31. Bacillary forms with unevenly distributed chromatin. Schu strain in culture. Hoffman violet.

32. Ring forms, free and filamentously connected. Note the M.R.U. along the filament and terminal budding at night. Russ strain in saline. Bismark brown.

33. Ring, bacillary, and filamented forms. Memp strain in culture. Bismark brown.

34. Ring and bacillary forms. Memp strain in culture. Bismark brown.

35. Ring forms and globules. Note dumbbell form at left, with a M.R.U. in the connecting filament and a sessile bud on the lower ring. Memp strain in culture. Bismark brown.

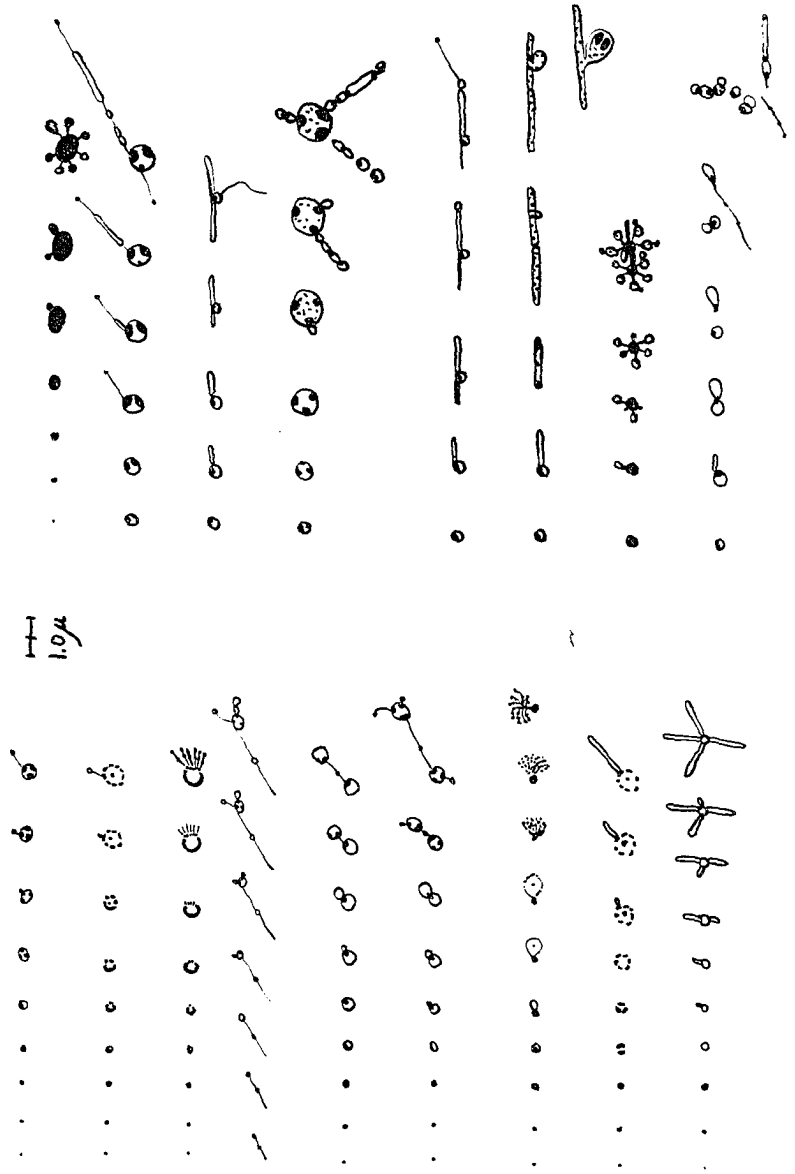

Fig. 5.

Figure 5.

Observed modes of development of Bacterium tularense as seen in dark field illumination of living cultures in the Tamura and Gibby gelatin hydrolyzate medium.

The left hand column, and the upper line of the right column, show the forms that grew from minimal reproductive units.

The rest of the right hand column shows additional forms that developed from ring or coccoid forms.

Table 1.

Agglutinin Titers in Tularemia Patients

y of Disease		1 : 10	20	40	80	160	320	640	1280	2560	5120
3rd	H	0	0	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
3rd	H	0	0	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
6th	H	0	0	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
6th	H	0	0	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
9th	H	4-3	3-2	21	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
10th	H	4-3	2-1	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
10th	H	4	4	4	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
11th	H	4	4	4	0	0	0	0	0	0	0
	O	2	0	0	0	0	0	0	0	0	0
12th	H	4	4	4	4	2	0	0	0	0	0
	O	2	0	0	0	0	0	0	0	0	0
12th	H	4	4	4	4	4	4	0	0	0	0
	O	4	0	0	0	0	0	0	0	0	0
12th	H	4	4	4	4	4	4	0	0	0	0
	O	4	4	4	0	0	0	0	0	0	0
13th	H	4-3	2-1	0	0	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
13th	H	4	4	4	4	4	4	4	4	4	0
	O	4	4	4	4	2	0	0	0	0	0
14th	H	4	4	4	4	4	2	0	0	0	0
	O	4	4	2	0	0	0	0	0	0	0
16th	H	4	4	4	4	4	4	4	4	4	0
	O	4	4	4	4	4	4-3	2-1	0	0	0
17th	H	4	4	4	4	0	0	0	0	0	0
	O	0	0	0	0	0	0	0	0	0	0
17th	H	4	4	4	4	4	4	4	0	0	0
	O	4	4	0	0	0	0	0	0	0	0
17th	H	4	4	4	4	4	4	4	4	0	0
	O	4	4	4	4	2	0	0	0	0	0
18th	H	4	4	4	4	4	0	0	0	0	0
	O	4	4	2	0	0	0	0	0	0	0

Day of Disease	1 :	10	20	40	80	160	320	640	1280	2560	5120
18th	H	4	4	4	4	4	4	4	4	2	0
	0	4	4	4	0	0	0	0	0	0	0
20th	H	4	4	4	4	4	4	4	2	0	0
	0	4	4	4	2	0	0	0	0	0	0
20th	H	4	4	4	4	4	4	4	4	4	0
	0	4	4	4	4	4	4	2	0	0	0
21st	H	4	4	4	4	4	4	4	0	0	0
	0	4	4	4	2	0	0	0	0	0	0
24th	H	4	4	4	4	4	4	4	0	0	0
	0	4	4	4	4	0	0	0	0	0	0
26th	H	4	4	4	4	4	4	4	2	0	0
	0	4	4	4	2	0	0	0	0	0	0
27th	H	4	4	4	4	4	4	4	4	4	0
	0	4	4	4	4	4	4	2	0	0	0
31st	H	4	4	4	4	4	4	4	4	0	0
	0	4	4	4	4	2	0	0	0	0	0
33rd	H	4	4	4	4	4	4	4	4	4	4
	0	4	4	4	4	4	4	2	0	0	0
34th	H	4	4	4	4	4	4	4	0	0	0
	0	4	4	4	4	0	0	0	0	0	0
41st	H	4	4	4	4	4	4	4	4	4	2
	0	4	4	4-3	2-1	0	0	0	0	0	0
46th	H	4	4	4	4	4	4	4	0	0	0
	0	4	4	4	0	0	0	0	0	0	0
52nd	H	4	4	4	4	4	0	0	0	0	0
	0	2	0	0	0	0	0	0	0	0	0
150th	H	4	4	4	4	4	4	2	0	0	0
	0	4	2	0	0	0	0	0	0	0	0

Table 2.
Agglutinin Titers in Vaccinated Persons

	Serum Dilutions 1: 10	20	40	80	160	320	640	1280	2560	Days after last injection
1.	H 4	4	3-2	0	0	0	0			8
	0 3-2	0	0	0	0	0	0			
2.	H 4	4	4	0	0	0				9
	0 0	0	0	0	0	0				
3.	H 3	0	0	0	0	0	0			10
	0 3	0	0	0	0	0	0			
4.	H 4	4	4	2	0	0				10
	0 1-0	0	0	0	0	0				
5.	H 4	4	4	4-3	0	0	0			10
	0 4	4-3	0	0	0	0	0			
6.	H 4	4	4	4	2	0				10
	0 0	0	0	0	0	0				
7.	H 4	4	4	4	3	2	0			10
	0 4	4	2	0	0	0	0			
8.	H 4	4	4	2	0	0				11
	0 2-1	0	0	0	0	0				
9.	H 4	4	4	2	0	0				12
	0 0	0	0	0	0	0				
10.	H 4	4	4	3	0	0	0			12
	0 2	0	0	0	0	0	0			
11.	H 4	4	4	4-3	0	0				12
	0 0	0	0	0	0	0				
12.	H 4	4	4	4	2	0	0			12
	0 4	4	2--1	0	0	0	0			
13.	H 0	0	0	0	0	0				13
	0 0	0	0	0	0	0				
14.	H 4	4	2-1	0	0	0				13
	0 1-0	0	0	0	0	0				
15.	H 4	4	4	2	0	0				13
	0 0	0	0	0	0	0				
16.	H 4	4	4	4	2	0	0			13
	0 4	4	3-2	0	0	0	0			
17.	H 4	4	4	4	4-3	0	0			13
	0 4	4-3	2	0	0	0	0			
18.	H 0	0	0	0	0	0				14
	0 0	0	0	0	0	0				
19.	H 4	2	0	0	0	0	0			14
	0 3	0	0	0	0	0	0			

	1: 10	20	40	80	160	320	640	1280	2560	Days after last injection
20.	H 4	4-3	1-0	0	0	0				14
	0 1-0	0	-0	0	0	0				
21.	H 4	4	3	2	0	0	0			14
	0 0	0	0	0	0	0	0			
22.	H 4	4	4	2	0	0				14
	0 1-0	0	0	0	0	0				
23.	H 4	4	4	2	0	0				14
	0 2	2	0	0	0	0				
24.	H 4	4	4	2	0	0	0			14
	0 4	2	0	0	0	0	0			
25.	H 4	4	4	4-3	0	0				14
	0 2	0	0	0	0	0				
26.	H 4	4	4	3	1	0	0			14
	0 0	0	0	0	0	0	0			
27.	H 4	4	4	3	2	0	0			14
	0 0	0	0	0	0	0	0			
28.	H 4	4	4	4	2	0				14
	0 2	0	0	0	0	0				
29.	H 4	4	4	4	2	0				14
	0 4	2	0	0	0	0				
30.	H 4	4	4	4	4-3	0	0			14
	0 4	4-3	0	0	0	0	0			
31.	H 4	4	4	4	3	2	0			14
	0 3	2	0	0	0	0	0			
32.	H 4	4	4	4	4	2				14
	0 3	2	0	0	0	0				
33.	H 4	4	4	4	4	2-1				14
	0 2	2-1	0	0	0	0				
34.	H 4	4	4	4	4-3	2				14
	0 4	4	2	0	0	0				
35.	H 4	4	4	4	3	2	2	0	0	14
	0 2	0	0	0	0	0	0	0	0	
36.	H 4	4	4	4	4	3	2			14
	0 2	0	0	0	0	0	0			
37.	H 0	0	0	0	0	0				15
	0 0	0	0	0	0	0				
38.	H 4	4	4	4	4	2-1	0			15
	0 4	4	2	0	0	0	0			
39.	H 4	4	4	4	4	2-1	0			16
	0 4	4	4	3-2	0	0	0			

	1: 10	20	40	80	160	320	640	1280	2560	Days after last injection
40.	H 2-1	0	0	0	0	0				17
	0 0	0	0	0	0	0				
41.	H 4	4	1-0	0	0	0				17
	0 0	0	0	0	0	0				
42.	H 4	4	4	3-2	0	0				17
	0 2	0	0	0	0	0				
43.	H 4	4	4	4	4	4-3				17
	0 4	3	2	0	0	0				
44.	H 4	4	4	4	4	4	4-3			17
	0 4	4	4	2	0	0	0			
45.	H 4	4	4	4	4	4	4	2	1	17
	0 4	4	3	1	1	0	0	0	0	
46.	H 4	4	2	0	0	0				18
	0 1-0	0	0	0	0	0				
47.	H 4	4	4	4	4-3	1	0			18
	0 4	2	0	0	0	0	0			
48.	H 4	4	4	0	0	0				19
	0 0	0	0	0	0	0				
49.	H 4	4	4	4	4	2-1				19
	0 4	4-3	1	0	0	0				
50.	H 4	4	4	2	0	0	0			20
	0 4	2	0	0	0	0	0			
51.	H 4	4	4	2	0	0	0			20
	0 4	2	0	0	0	0	0			
52.	H 4	4	4-3	0	0	0	0			21
	0 4	2-1	0	0	0	0	0			
53.	H 4	4	3	2	0	0	0			22
	0 4	2	0	0	0	0	0			
54.	H 4	4	4	3	2	0	0			22
	0 0	0	0	0	0	0	0			
55.	H 2	0	0	0	0	0				23
	0 0	0	0	0	0	0				
56.	H 4	4	4	4	4	4	2-1			23
	0 4	4	4	4	4-3	0	0			
57.	H 4	4	4	2	0	0	0			25
	0 4	2	0	0	0	0	0			
58.	H 4	4	4	4	2	0	0			25
	0 4	4	2-1	0	0	0	0			
59.	H 4	4	4	4	4-3	0	0			26
	0 4	4-3	0	0	0	0	0			

	1: 10	20	40	80	160	320	640	1280	2560	Days after last injection
60.	H 4	3-2	0	0	0	0				27
	0 0	0	0	0	0	0				
61.	H 4	4	4-3	0	0	0	0			28
	0 3-2	0	0	0	0	0	0			
62.	H 4	4	4	4	2	0	0			31
	0									
63.	H 4	4	4-3	2-1	0	0	0			32
	0 2-1	0	0	0	0	0	0			
64.	H 4	4-3	0	0	0	0	0			38
	0 0	0	0	0	0	0	0			
65.	H 4	4-3	0	0	0	0	0			44
	0 4	2	0	0	0	0	0			

HISTORIES OF THE STRAINS EXAMINED

Designation of culture	Year of original isolation	Pathologic source	Geographic source	Interval between last animal isolation and our examinations
38	1920	Human Ly. node	Utah	22 years
26	1921	*Human blood*	*Utah*	21 "
SF	1922	Ground squirrel	Calif.	20 "
T	1923	Wood tick	Montana	19 "
V	1924	Human spleen	D. C.	11 "
Sn	1925	Wild hare	Montana	17 "
Jap	1926	Human Ly. node	Japan	16 "
LR	1927	Human ascitic Fl.	Arkansas	15 "
Max	1928	Human Ly. node	Russia	14 "
Russ	1928	" " "	"	14 "
Can	1930	Wild hare	Canada	12 "
Ohara	1931	Unspecified	Japan	11 "
Ri	1932	Human pus	Virginia	10 "
Fox I	1933	Gray fox spleen	Minnesota	9 "
Col	1933	Human pleural Fl.	So. Car.	8 "
LI	1934	Human pus	Canada	8 "
Tol	1934	Human blood	Ohio	7 "
T-418	1934	Human blood	Ohio	6 "
L.C.	1935	Human ulcer	Virginia	7 "
H.D.	1935	Human eye	Austria	7 "
Md	1936	Human pleura	Maryland	6 "
P F	1936	Human lung	Ohio	2 "
Chr	1937	Human pus	Ohio	12 days
Sto	1937	Human blood	Ohio	5 years
Chil	1937	Human pus	Ohio	4 "
Trot	1937	Human lung	Ohio	8 months
Die	1938	Human ulcer	Calif.	2 years
Memp	1938	Human Ly. node	Tenn.	10 days
De P	1938	" " "	Ohio	2 years
Pi	1938	Human blood	Ohio	8 days
Pack	1939	Human sputum	D. C.	2 years
V. F.	1940	Human pleural Fl.	Tenn.	2 "
Hugh	1940	Human ulcer	Ohio	2 "
Clem	1940	Human blood	Ohio	2 "
Fox	1940	" "	Ohio	1 "
Broo	1941	Human spleen	New York	1 "
Fish	1941	Human ulcer	Ohio	10 months
Well	1941	Human pus	Ohio	9 "
Fran	1941	Human sputum	Ohio	5 "
Gib	1941	Human ulcer	Ohio	3 "
Schu	1941	" "	Ohio	3 days
Chur	1941	Human lung	Ohio	2 "
Bish	1942	Human pleural Fl.	Tenn.	4 months

Table 3.

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